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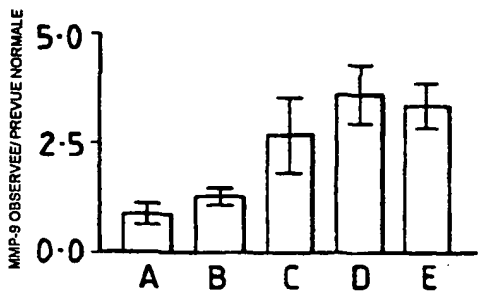
(54) Title: NOVEL ASSAY



(57) Abstract: The present invention provides an assay for the detection of a malignant pathology such as colorectal cancer. The assay comprises the steps of measuring a value for the level of a first matrix metalloproteinase (MMP) in a blood serum sample obtained from a patient, the MMP being one whose level is altered by the malignant pathology, calibrating the value, and comparing the calibrated value with a reference value. Deviation of the calibrated value from the reference value is indicative of the malignant pathology in the patient.

NOVEL ASSAY

The present invention relates to an assay for the detection of a malignant pathology in a patient.

A significant factor affecting the long term survival of cancer patients is the stage at which the cancer is detected. Early detection facilitates rapid and complete removal of any malignancy before metastasis occurs and correlates well with increased cure rate and long-term survival. Perhaps the best method of early detection is routine screening of those considered to be at risk. For example, visualisation of pre-cancerous cervical cells (smear test) is an early indicator of potential cervical cancer in women. In men, elevated levels of prostate specific antigen (PSA) is a possible indication of prostate cancer.

Existing screening methods have met with variable success. They may be too expensive and/or unreliable to be widely used. In any case, for many cancers, there is no practical screening method available. The problem is compounded by the fact that certain cancers, eg. gastric and colorectal cancers, exhibit symptoms consistent with much more common non-malignant pathologies, making them difficult to diagnose at an early stage. Currently, the only way of diagnosing colorectal cancer is to carry out a colonoscopy. This is impractical as a screening technique for general applicability.

Thus, it is an object of the present invention to provide an assay for the detection of a malignant pathology in a patient which obviates or mitigates

one or more of the above-mentioned disadvantages and, in particular, which is suitable as a screening method to facilitate the early diagnosis of a malignant pathology.

According to the present invention, there is provided an assay for the detection of a malignant pathology in a patient comprising the steps of:-

(i) measuring a value for the level of a first matrix metalloproteinase (MMP) in a blood serum sample obtained from said patient, said MMP being one whose level is altered by the malignant pathology,

(ii) calibrating said value, and

(iii) comparing said calibrated value with a reference value

wherein deviation of said calibrated value from said reference value is indicative of said malignant pathology in said patient.

As used herein, malignant pathology is intended to refer to any condition which may be expected to progress to malignancy without intervention. Thus, it is within the scope of the present invention for said malignant pathology to be detected at a pre-malignant stage, i.e. prior to the development of any malignancy, for example to detect the existence of colorectal adenomas (polyps).

MMPs are zinc-dependent endopeptidases catalysing dissolution of the extracellular matrix. More than sixteen human MMPs have been identified to date, classified according to domain structure into collagenases, gelatinases, stromolysins, membrane-type and others. A physiological role exists for MMPs in tissue remodelling during growth phases and following injury. Tissues also show elevated levels in pathological processes

including chronic inflammatory processes and malignancy. Elevated MMP levels (of various sub-types) in human cancer tissue from breast, prostatic, gastric, ovarian and colorectal disease have been reported, leading to interest in the development of MMP inhibitors as potential tumour-control agents. In addition, it is known that MMP-9 is elevated in patients suffering gastric cancer. However, to the best of the inventors' knowledge, it has not previously been recognised that variations in blood serum MMP levels may provide the basis for a cancer screen. One possible reason for this is that there is a wide distribution of normal levels of MMP-9, making a determination of deviation from the norm difficult.

Preferably, said malignant pathology is colorectal, gastric, ovarian, cervical, breast, testicular or lung cancer, and more preferably colorectal, gastric, ovarian and cervical cancer.

It will be understood that said method is particularly suitable as an initial malignancy screen or as post-operative surveillance of cancer patients.

Preferably, said first MMP measured in step (i) is MMP-9 in which case malignant pathology is indicated when the calibrated value is higher than the reference value.

In a first embodiment, step (ii) is effected by dividing the measured MMP value by a normal age-corrected value to arrive at the calibrated value. A statistically significant higher calibrated value over the reference value (in this case 1) is indicative of the malignant pathology.

The inventors have found that there is a strong correlation of normal MMP-9 levels with age, thereby increasing the ability to measure deviation from the normal value.

In addition to natural variability, MMP levels may vary from the expected normal value for reasons unrelated to the presence of a malignant pathology, thereby giving rise to the possibility of false positive indications of malignant pathology. For example, MMP levels may be increased by inflammatory disorders, childbirth and wound repair.

Thus, in a second embodiment, step (ii) is effected by obtaining a ratio between the measured first MMP value and a value for a second MMP of a different sub-type obtained from the same blood serum sample, the second MMP being one whose level is substantially unaffected by the malignant pathology. The comparison of step (iii) is then made between the ratio obtained in step (ii) and a corresponding ratio obtained between a normal value of the first MMP and a normal value for the second MMP.

It has been found that the use of such ratios reduces variability in the assay produced by non-malignant conditions.

Preferably, said first MMP is MMP-9 (total or active) and said second MMP is MMP-2 (total or active). More preferably said first MMP-9 is total MMP-9 and said second MMP is active MMP-2. As used herein, "total MMP" is defined as active MMP and pre-processed MMP.

The ratio used may be MMP-9/MMP-2 (eg. colorectal cancer) or MMP-2/MMP-9 (e.g. breast cancer).

An embodiment of the present invention will now be described with reference to the accompanying drawings in which:-
Fig 1 is a plot of serum MMP-9 level against age for healthy controls, and
Fig 2 is a plot of the ratio of observed MMP-9 to age-corrected normal MMP-9 for different pathologies and a normal control.

Blood was collected from patients and control volunteers. After clot formation, serum was isolated by centrifugation and aliquots were frozen at –80°C. MMP-9 levels were measured using an enzyme-linked immunosorbent assay (ELISA) (DMP900 supplied by R&D systems) or an immunocapture activity assay (RPN2630 and RPN2631 for MMP-2 and MMP-9, respectively, from Amersham). The values obtained from 240 samples were compared to a predicted normal value for an age matched cohort. The predicted normal value was obtained by grouping the normal controls into age bands of 20-39 years, 40-59 years, 60-79 years and over 79 years. The mean MMP-9 level for each age group was plotted against the midpoint of that age band (i.e. 30, 50, 70 and 90). Linear regression provided a line equation: $-(7.579 \times \text{age}) + 798$ ($r^2 = 0.9913$) from which the predicted normal value could be calculated for any age.

Referring to Fig 2, the ratio of the measured value to the age predicted value was plotted. The following key is used:-
bar A: normal healthy volunteers,

bar B: patients with non-neoplastic pathologies (eg. haemorrhoids, diverticular disease and colitis),

bar C: patients exhibiting pre-malignant adenomatous disease (generally leading to colorectal carcinomas),

bar D: patients exhibiting colorectal carcinomas, and

bar E: sum of bars C and D.

As would be expected, the ratio of measured MMP-9 to the predicted normal value was close to 1 (mean = 0.8761) for samples from the healthy volunteers (bar A) and the samples from the patients exhibiting non-neoplastic pathologies showed no significant difference ($p = 0.0285$, bar B, mean = 1.271). Samples from patients diagnosed as suffering from colorectal cancer showed significant elevation in MMP-9 levels ($p < 0.0001$, bar D, mean = 3.581).

Surprisingly, patients exhibiting colorectal adenoma (polyps) also showed significant elevation of MMP-9 ($p < 0.0001$, bar C, mean = 2.661).

The adenoma-carcinoma sequence in colorectal neoplastic progression is widely recognised. It is generally accepted that an early event , which may be a mutation in the Adenomatous Polyposis Coli (APC) gene, induces polyp formation. The subsequent change in crypt architecture exposes areas that could act as foci for further mutations, resulting in eventual carcinogenesis. The process can take up to 15 years to develop from normal mucosa, in keeping with the increasing incidence of colorectal cancer with advancing age. Polyp excision is a relatively simple surgical procedure and dramatically reduces the progression to carcinoma.

Thus, the method of the present invention offers the prospect of identifying those at risk from colorectal cancer at an early stage by screening either (i) patients presenting at clinics with colorectal symptoms or (ii) general populations, ultimately reducing the cost to health authorities and offering patients an improved prognosis. That MMP-9 serum levels are elevated in pre-malignant colorectal patients is surprising since MMP-9 is not elevated in the polyp tissue itself.

As can be seen from the table below, the above-described ELISA derived a correct indication as to the presence or absence of neoplasia in 65.8% of samples. The false negative result (FNR) for prediction of neoplasia was 1.25%. Neoplasia was correctly predicted in 95.7% of samples, with 96.8% of negative sample ELISAs being correct.

	neoplastic	non-neoplastic	
ELISA +'ve[1]	67	79	146
ELISA –'ve[1]	3	91	94
	70	170	240

[1]An ELISA was regarded as positive when the MMP-9 value was greater than a cutoff of -(7.579 x age of patient) + 798 ng/ml.

In another experiment, zymograms (not shown) were made of MMP-9 and MMP-2 activity from serum obtained from a normal control and six patients afflicted with different cancers (colorectal, prostatic, pancreatic, breast, gastric and ovarian). In all the cancer types, MMP-9 gelatin degradation was higher than the control. In the non-colonic cancers, the MMP-2 gelatin degradation was also higher than the control but the colorectal cancer was not increased relative to the control. This suggests

qualitatively that a screen based on the ratio of MMP-9/MMP-2 may be colorectal cancer specific.

CLAIMS

1. An assay for the detection of a malignant pathology in a patient comprising the steps of:-

(i) measuring a value for the level of a first matrix metalloproteinase (MMP) in a blood serum sample obtained from said patient, said MMP being one whose level is altered by the malignant pathology,

(ii) calibrating said value, and

(iii) comparing said calibrated value with a reference value wherein deviation of said calibrated value from said reference value is indicative of ~~said malignant pathology in said patient.~~

2. As assay as claimed in claim 1 for the detection of a malignant pathology selected from the group consisting of colorectal, gastric, ovarian, cervical, breast, testicular and lung cancer.

3. An assay as claimed in claim 2, wherein said malignant pathology is selected from the group consisting of colorectal, gastric, ovarian and cervical cancer.

4. An assay as claimed in any preceding claim, wherein said first MMP measured in step (i) is MMP-9.

5. An assay as claimed in any preceding claim, wherein step (ii) is effected by dividing the measured MMP value by a normal age-corrected value to arrive at the calibrated value.

6. An assay as claimed in any one of claims 1 to 3, wherein step (ii) is effected by obtaining a ratio between the measured first MMP value and a value for a second MMP of a different sub-type obtained from the same blood serum sample, the second MMP being one whose level is substantially unaffected by the malignant pathology and wherein the comparison of step (iii) is made between the ratio obtained in step (ii) and a corresponding ratio obtained between a normal value of the first MMP and a normal value for the second MMP.

7. An assay as claimed in claim 6, wherein said first MMP is MMP-9 ~~(total or active) and said second MMP is MMP-2 (total or active).~~

8. An assay as claimed in claim 7, wherein said first MMP-9 is total MMP-9 and said second MMP is active MMP-2.

9. An assay as claimed in claim 7 or 8, wherein the ratios used in steps (ii) and (iii) are MMP-9/MMP-2.

10. An assay as claimed in claim 7 or 8, wherein the ratios used in steps (ii) and (iii) are MMP-2/MMP-9.

11. A diagnostic kit adapted and constructed for carrying out the assay of any one of claims 1 to 10, said kit comprising means for measuring a valve for the level of a first MMP in a blood serum sample obtained from a patient.

12. A diagnostic kit in accordance with claim 11 including instructions for carrying out the assay of any one of claims 1 to 10.

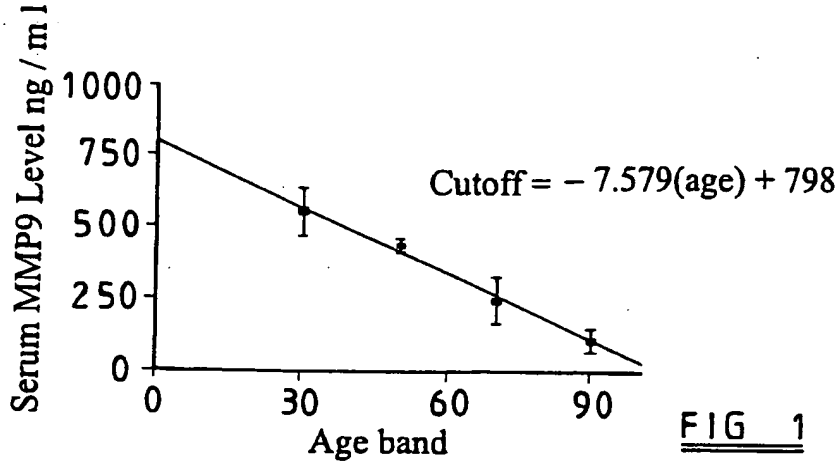

Serum MMP9 Level ng / m l
1000
750
500
250
0
0
30
60
90
Age band
Cutoff = – 7.579(age) + 798

FIG 1

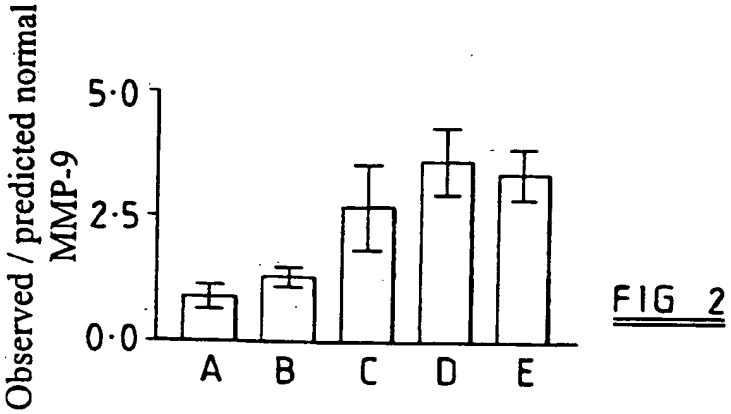

Observed / predicted normal MMP-9
5·0
2·5
0·0
A
B
C
D
E

FIG 2